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THE PLATELET FUNCTION DEFECT OF CARDIOPULMONARY BYPASS

BY

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The use of cardiopulmonary bypass (CPB) during cardiac surgery is associated with a hemostatic defect, the hallmark of which is a markedly prolonged bleeding time. However, the nature of the putative platelet function defect is controversial. In this study, blood from 16 patients was collected and analyzed at 10 time points before, during and after CPB. We utilized a whole blood flow cytometric assay to study platelet surface glycoproteins in a) peripheral blood, b) peripheral blood activated in vitro by either phorbol

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myristate acetate, the thromboxane (TX) A2 analogue U46619, or a combination of adenosine diphosphate and epinephrine, and c) the blood emerging from a bleeding time wound (shed blood). Activation-dependent changes were detected by monoclonal antibodies directed against GPIb, the GPIIb-IIIa complex, and P-selectin. In addition, we measured plasma glycocalicin (a proteolytic fragment of GPIb) and shed blood TXB2 (a stable breakdown product of TXA2). In shed blood emerging from a bleeding time wound, the usual time-dependent increase in platelet surface P-selectin was absent during CPB, but returned to normal within 2 hours. This abnormality paralleled both the CPB-induced prolongation of the bleeding time and a CPB-induced marked reduction in shed blood TXB2 generation. In contrast, there was no loss of platelet reactivity to in vitro agonists during or after CPB. In peripheral blood, platelet surface P-selectin was negligible at every time point, demonstrating that CPB resulted in virtually no circulating degranulated platelets. CPB did not change the platelet surface expression of GPIb in peripheral blood, as determined by the platelet binding of a penel of monoclonal antibodies, ristocetin-induced binding of von Willebrand factor, and a lack of increase in plasma glycocalicin. CPB did not change the platelet surface expression of the GPIIb-IIIa complex in peripheral blood, as determined by the platelet binding of fibrinogen and a panel of monoclonal antibodies. In summary, CPB resulted in: 1) no loss of the platelet surface GPIb-IX complex, 2) no loss of the platelet surface GPIIb-IIIa complex, 3) a minimal number of circulating degranulated platelets, 4) normal platelet reactivity in vitro, and 5) markedly deficient platelet reactivity in vivo. These data suggest that the "platelet function defect" of CPB is not a defect intrinsic to the platelet but is an extrinsic defect such as an in vivo lack of availability of platelet agonists. The near universal use of heparin during CPB is likely to contribute substantially to this defect via its inhibition of thrombin, the preeminent platelet activator.

ABSTRACT

The use of cardiopulmonary bypass (CPB) during cardiac surgery is associated with a hemostatic defect, the hallmark of which is a markedly prolonged bleeding time. However, the nature of the putative platelet function defect is controversial. In this study, blood from 16 patients was collected and analyzed at 10 time points before, during and after CPB. We utilized a whole blood flow cytometric assay to study platelet surface glycoproteins in a) peripheral blood, b) peripheral blood activated *in vitro* by either phorbol myristate acetate, the thromboxane (TX) A_2 analogue U46619, or a combination of adenosine diphosphate and epinephrine, and c) the blood emerging from a bleeding time wound (shed blood). Activation-dependent changes were detected by monoclonal antibodies directed against GPIb, the GPIIb-IIIa complex, and P-selectin. In addition, we measured plasma glycocalicin (a proteolytic fragment of GPIb) and shed blood TXB_2 (a stable breakdown product of TXA_2). In shed blood emerging from a bleeding time wound, the usual time-dependent increase in platelet surface P-selectin was absent during CPB, but returned to normal within 2 hours. This abnormality paralleled both the CPB-induced prolongation of the bleeding time and a CPB-induced marked reduction in shed blood TXB_2 generation. In contrast, there was no loss of platelet reactivity to *in vitro* agonists during or after CPB. In peripheral blood, platelet surface P-selectin was negligible at every time point, demonstrating that CPB resulted in virtually no circulating degranulated platelets. CPB did not change the platelet surface expression of GPIb in peripheral blood, as determined by the platelet binding of a panel of monoclonal antibodies, ristocetin-induced binding of von Willebrand factor, and a lack of increase in plasma glycocalicin. CPB did not change the platelet surface expression of the GPIIb-IIIa complex in peripheral blood, as determined by the platelet binding of fibrinogen and a panel of monoclonal antibodies. In

summary, CPB resulted in: 1) no loss of the platelet surface GPIb-IX complex, 2) no loss of the platelet surface GPIIb-IIIa complex, 3) a minimal number of circulating degranulated platelets, 4) normal platelet reactivity *in vitro*, and 5) markedly deficient platelet reactivity *in vivo*. These data suggest that the "platelet function defect" of CPB is not a defect intrinsic to the platelet but is an extrinsic defect such as an *in vivo* lack of availability of platelet agonists. The near universal use of heparin during CPB is likely to contribute substantially to this defect via its inhibition of thrombin, the preeminent platelet activator.

INTRODUCTION

The use of cardiopulmonary bypass (CPB) during cardiac surgery is associated with a generalized hemorrhagic defect.[1] Although thrombocytopenia of mild degree and alterations in the fibrinolytic and coagulation systems occur during CPB,[1] a platelet function defect is generally considered to be the primary CPB-induced hemostatic deficit.[1-3] CPB consistently results in a reversible marked prolongation of the bleeding time[1-4] and many,[4-11] but not all,[12,13] studies have reported defects in *in vitro* platelet aggregation. However, the nature of the putative platelet function defect associated with CPB remains controversial. Three types of intrinsic platelet defects have been reported in association with CPB. First, some,[4,7,14] but not other,[15-18] studies have reported that CPB results in partial platelet degranulation. Second, some,[7,17,19] but not all,[20] studies have reported CPB-induced defects in the platelet surface glycoprotein (GP) Ib-IX complex. Third, some,[7,13,16,17] but not other,[18,19] studies have reported defects in the platelet surface GPIIb-IIIa complex. In the present study, whole blood flow cytometry was utilized to circumvent many of the potential methodologic problems of other assays. Evidence is presented that the CPB-induced platelet dysfunction is the result of a defect extrinsic to the platelet.

METHODS

Study Population

The study was approved by the Human Subjects Committee of the Brockton\West Roxbury Veterans Administration Medical Center. Twenty patients with angiographically documented coronary artery disease referred to the Brockton\West Roxbury Veterans Administration Medical Center for coronary artery revascularization were entered into the study after written informed consent was obtained. All patients were undergoing their first open heart procedure. All patients required coronary artery bypass grafting, while one required concomitant valvular replacement. No patient had a history suggestive of an underlying hemostatic disorder. The age of the subjects in the study was 62 $\pm$ 2.3 years (mean $\pm$ S.E.M., n = 20). Nineteen of the 20 subjects were male. All patients underwent a standard CPB procedure using a membrane oxygenator. The mean aortic cross-clamp time was 50.4 $\pm$ 4.4 minutes and the mean duration of CPB was 109.9 $\pm$ 7.2 minutes. Patients received intravenous heparin at an initial dose of 4 mg/kg, followed by additional doses as necessary to maintain the activated clotting time (ACT) greater than or equal to 999 seconds. At the completion of CPB, heparin was reversed with protamine sulfate. Heparin reversal was verified by confirming that the ACT had returned to the preoperative value. Maximal hypothermia during CPB was a core temperature of 26.8 $\pm$ 1.0 degrees Celsius, as determined by a bladder thermometer (Bard, Boston, MA). Seven patients required no exogenous blood components intraoperatively, but most patients received autologous blood harvested intraoperatively using a cell saver (Haemonetics Corp., Braintree, MA). None of the patients had excessive perioperative bleeding, as defined by transfusion of greater than 10 units of blood in the perioperative period.[1] The number of units of packed red blood cells transfused in the perioperative period was 1.8 $\pm$ 0.4 (mean $\pm$ S.E.M.). The

maximum number of units of packed red blood cells transfused into any one patient was 6 and this patient was found to have surgical bleeding. Data from this patient did not differ statistically from that of the other subjects and was therefore included in the analysis. No patient received a platelet transfusion before or during CPB.

Blood Sampling Time Points

Peripheral venous blood and the shed blood emerging from a standardized bleeding time wound (Simplate II, Organon Teknika, Durham NC) were collected before, during, and after CPB at the time points listed in Table 1. Not every patient had all studies performed at all time points.

Murine Monoclonal Antibodies

S12 (provided by Dr. Rodger P. McEver, University of Oklahoma) is a monoclonal antibody directed against P-selectin.[21,22] P-selectin, also referred to as GMP-140,[21] platelet activation-dependent granule-external membrane (PADGEM) protein,[23] and CD62,[24] is a component of the α granule membrane of resting platelets that is only expressed on the platelet plasma membrane after platelet secretion.[25]

A panel of platelet GPIb-IX-specific monoclonal antibodies was utilized. 6D1 (provided by Dr. Barry S. Coller, SUNY, Stony Brook) is directed against the von Willebrand factor binding site on the glycocalicin portion of the α-chain of GPIb.[26,27] TM60 (provided by Dr. Naomasa Yamamoto, Tokyo Metropolitan Institute of Medical Science) is directed against the thrombin binding site on the amino terminal domain of the α chain of GPIb.[28-30] AK1 (provided by Dr. Michael C. Berndt, Baker Institute, Melbourne, Australia) is directed against the GPIb-IX complex.[31] AK1 only

binds to the intact GPIb-IX complex, not to uncomplexed GPIb or GPIX.[31] FMC25 (provided by Dr. Berndt) is directed against GPIX.[32,33]

A panel of platelet GPIIb-IIIa-specific monoclonal antibodies was utilized. Y2/51 (DAKO, Carpinteria, CA) is directed against platelet membrane GPIIIa.[34] 7E3 (provided by Dr. Coller), 10E5 (provided by Dr. Coller), and M148 (Cymbus Bioscience, Southampton, U.K.) are directed against different epitopes near the fibrinogen binding site on the GPIIb-IIIa complex.[35-38] PAC1 (provided by Dr. Sanford J. Shattil, University of Pennsylvania, Philadelphia) is directed against the fibrinogen binding site exposed on the GPIIb-IIIa complex of activated platelets.[39] Unlike Y2/51, M148, 7E3, and 10E5,[35-37] PAC1 does not bind to resting platelets.[39]

OKM5 (provided by Dr. Patricia Rao, Ortho Diagnostic Systems, Raritan, NJ) is directed against platelet membrane GPIV.[40]

Flow Cytometric Analysis of Platelet Surface Glycoproteins in Peripheral Blood

A whole blood flow cytometric method was utilized. The method has previously been described in detail[41] and includes no centrifugation, gel filtration, vortexing, or stirring steps that could artefactually activate platelets. Briefly, the method was as follows. The first 2 mL of blood drawn were discarded and then blood was drawn into a sodium citrate Vacutainer (Becton Dickinson, Rutherford, NJ). Within 15 minutes, the anticoagulated blood was: a) diluted in modified Tyrode's buffer (137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 0.35% bovine serum albumin, 10 mM HEPES, 5.5 mM glucose, pH 7.4), and b) incubated at 22°C with an agonist (either phorbol myristate acetate [PMA] [Sigma, St. Louis, MO], the stable thromboxane A_2 analogue U46619 [Cayman Chemical, Ann Arbor, MI], purified human α-thrombin [provided by Dr. John Fenton II, New York Department of Health, Albany] together with 2.5 mM glycyl-L-

prolyl-L-arginyl-L-proline [Calbiochem, San Diego, CA] [an inhibitor of fibrin polymerization[41]], or a combination of adenosine diphosphate [ADP] [Bio/Data Corp., Hatboro, PA] and epinephrine [Sigma]) or buffer only. In kinetic studies, the samples were then fixed with 1% formaldehyde at various time points after the addition of the agonist. In all other studies, fixation was performed 15 minutes after the addition of the agonist, as previously described.[41] After fixation, all samples were diluted and incubated at 22°C for 15 minutes with a) a saturating concentration of a biotinylated monoclonal antibody (directed against either P-selectin, the GPIIb-IIIa complex, or the GPIb-IX complex) and b) a saturating concentration of either fluorescein isothiocyanate (FITC)-conjugated GPIV-specific monoclonal antibody OKM5 or FITC-conjugated GPIIIa-specific monoclonal antibody Y2/51. The samples were then incubated at 22°C for 15 minutes with phycoerythrin-streptavidin (Jackson ImmunoResearch, West Grove, PA). (When monoclonal antibody M148 was used, the incubation step with phycoerythrin-streptavidin was unnecessary because the antibody was purchased directly conjugated with R-phycoerythrin.) Within 24 hours of antibody tagging, the samples were analyzed in an EPICS Profile flow cytometer (Coulter Cytometry, Hialeah, FL). After identification of platelets by gating on both FITC positivity and their characteristic light scatter, binding of the biotinylated monoclonal antibody was determined by analyzing 5,000 individual platelets for phycoerythrin fluorescence. In addition to platelets, OKM5 binds to monocytes[40] but these were completely gated out by size (forward light scatter). We[41] and others[42] have shown that this method results in no significant differences in fluorescence intensity between samples analyzed immediately and samples analyzed within 24 hours of antibody tagging. In addition to our standard method of determination of binding by fluorescence intensity relative to a maximally degranulated platelet,[41] some samples were analyzed, as indicated in Results, by the percentage of P-selectin-positive platelets. The percentage of P-selectin-positive platelets at different time points during CPB was

defined as the percentage of platelets that had an S12 fluorescence greater than 98% of the platelets in samples from the preoperative time point (without an added exogenous platelet agonist).

In a separate subgroup of patients, the response of washed platelets to thrombin was studied. Peripheral blood was collected as described above and the platelets separated and washed in modified Tyrode's buffer as previously described.[43] The platelets (75,000/μL) were incubated with 0.05, 0.1, or 1 U/mL of purified human α-thrombin or buffer only, and then analyzed by flow cytometry for the binding of a biotinylated monoclonal antibody (either S12, PAC1, 7E3, or 6D1) as previously described.[44]

Flow Cytometric Analysis of Platelet Surface P-selectin in Shed Blood

The platelet surface expression of P-selectin in blood emerging from a standardized bleeding time wound was analyzed by the whole blood flow cytometric method. The method has been previously described in detail.[41] Duplicate standardized bleeding times were performed on the forearm of patients with a Simplate II device (General Diagnostics, Durham, NC). The blood emerging from the bleeding time wound (shed blood) was collected with a micropipet at 2 minute intervals until the bleeding stopped. After each pipetting, any residual blood at the bleeding time wound site was removed with filter paper. Immediately after collection at each time point, the pipetted blood was added to a microfuge tube containing sodium citrate, fixed for 30 minutes at 22°C with an equal volume of 2% formaldehyde, and diluted 1:10 by volume in modified Tyrode's buffer. As described above, the fixed diluted whole blood samples were then labeled with the FITC-conjugated GPIV-specific monoclonal antibody OKM5 and the biotinylated P-selectin-specific monoclonal antibody S12, and the individual platelets analyzed in an EPICS Profile flow cytometer to assess S12 binding.

Plasma Glycocalicin Assay

Plasma glycocalicin was determined by a modified version of a previously described competitive inhibition assay.[45,46] A subsaturating concentration of FITC-conjugated monoclonal antibody 6D1 (1.2 μg/mL) was incubated at 22°C for 20 minutes with either: a) test plasma that had been filtered through a 0.22 μm Acrodisc (Gelman, Ann Arbor, MI) and the pH buffered to 7.4; or b) various concentrations of purified glycocalicin (prepared as previously described[27]). Samples were then incubated at 22°C for 20 minutes with fixed, washed platelets (final concentration 100,000/μL) and diluted 20-fold in modified Tyrode's buffer, pH 7.4 before the platelet binding of 6D1 was analyzed by flow cytometry. Linear regression analysis was used to generate a standard curve from 0 to 70 nM from the purified glycocalicin samples. The plasma glycocalicin concentration of unknown plasma samples was derived from this standard curve.

Ristocetin-induced Binding of von Willebrand Factor to Platelets

The ristocetin-induced binding of normal von Willebrand factor to washed platelets from the patient was determined by the following method. The patient's platelets were washed as previously described,[43] fixed in 1% formaldehyde, and resuspended at a concentration of 75,000/μL in modified Tyrode's buffer, pH 7.4. Twenty μL of the platelet suspension was incubated at 22°C for 15 minutes with 20 μL of pooled platelet-poor plasma from normal donors (as a source of von Willebrand factor) and 5 μL of ristocetin (BioData, Horsham, PA) (final concentration 1.4 mg/mL). The mixture was then incubated at 22°C for 15 minutes with 0.028 mg/mL of either polyclonal FITC-conjugated anti-von Willebrand factor goat IgG antibody (Atlantic Antibodies, Stillwater, MN) or FITC-conjugated non-specific goat IgG (Atlantic Antibodies). The sample was then diluted 16-fold in modified Tyrode's buffer, pH 7.4 and analyzed for green fluorescence by flow cytometry.

The fluorescence of the sample incubated with the non-specific goat IgG was subtracted from the sample incubated with the anti-von Willebrand factor antibody.

von Willebrand Factor Assay

The plasma concentration of von Willebrand factor was determined by an enzyme-linked immunosorbent assay (ELISA) kit (Asserachrom vWF, Diagnostica Stago, Asnieres-Sur-Seine, France).

Fibrinogen Binding to Platelets

Fibrinogen binding to ADP-stimulated washed platelets was determined by a direct binding assay using radioiodinated fibrinogen, as previously described.[47]

Radioimmunoassay of Shed Blood Thromboxane B_2

The method has been previously described.[48] A standardized bleeding time wound was performed, as described above. The shed blood emerging from the wound was aspirated through a blunt needle into a tuberculin syringe coated with heparin (1,000 U/mL) and containing 20 μL of ibuprofen for each 1 mL of blood (1.9 mg/mL). Samples were collected every 30 seconds until a 600 μL aliquot of blood was obtained. The thromboxane B_2 concentration of the aliquot was determined with an RIA kit (New England Nuclear, Boston, MA).

Hematocrit and Platelet Count

Hematocrit and platelet counts were measured using a Coulter J.T. Electronic Particle Counter (Coulter Electronics, Hialeah, FL).

Statistical Analysis

All statistical analyses were performed using Systat (Systat Inc., Evanston, IL) version 5.02. The reported p values were generated using either paired Student's t-test analysis or ANOVA for repeated measures, as indicated. Unless otherwise stated, all p values were generated by comparing measurements of a given parameter in blood obtained during bypass to measurements of the same parameter before and after bypass. Statistical significance was assumed for p values less than or equal to 0.05.

RESULTS

Effect of CPB on the Bleeding Time and Platelet Count

The bleeding time was modestly prolonged by heparin administration (10.4 $\pm$ 0.5 minutes) (mean $\pm$ S.E.M., n = 16), markedly prolonged during CPB (33.6 $\pm$ 3.7 minutes), and again only modestly prolonged immediately after the completion of CPB (Fig 1, panel A).

A platelet function defect appeared to be the basis for this marked prolongation of the bleeding time during CPB, because a) the platelet count declined to 166.1 $\pm$ 12.7 x $10^3/\mu L$ after the start of CPB and remained at only marginally thrombocytopenic levels at all time points up to and including 24 hours after the completion of CPB (Fig 1, panel B), and b) von Willebrand factor levels remained within or above the normal range at all time points (Fig 1, panel C).

Effect of CPB on the Platelet Surface GPIb-IX Complex

The platelet surface expression of the GPIb-IX complex can be markedly reduced by platelet activation[41,43,49] and washing.[50] In order to avoid these potential artifacts, we utilized a whole blood flow cytometric method that did not require any centrifugation, gel filtration, vortexing, or stirring steps.[41] As determined by this method, CPB did not result in significant change in the platelet surface expression of the GPIb-IX complex, irrespective of whether the monoclonal antibody utilized was directed against the von Willebrand factor binding site on GPIb (Fig 2, panel A), the thrombin binding site on GPIb (Fig 2, panel B), GPIX (Fig 2, panel C), or the GPIb-IX complex (Fig 2, panel D).

In addition, we examined the ristocetin-induced binding of von Willebrand factor to platelets. This binding, which reflects the availability of GPIb as a platelet surface receptor for von Willebrand factor,[51,52] was unchanged during and after CPB (Fig 2, panel E).

Furthermore, we assayed the patients' plasma for glycocalicin, a proteolytic product of GPIb.[53,54] Plasma glycocalicin did not rise above the preoperative concentration at any time point during or after CPB (Fig 3). The observed reduction in plasma glycocalicin during CPB was probably dilutional because the reduction paralleled the reductions in hematocrit (Fig 3) and serum albumin (data not shown).

In summary, these data demonstrate that CPB did not result in a loss of the platelet surface GPIb-IX complex, as determined by a) a panel of GPIb-IX-specific monoclonal antibodies in a whole blood assay, b) ristocetin-induced binding of von Willebrand factor, and c) plasma glycocalicin.

Effect of CPB on the Platelet Surface GPIIb-IIIa Complex

The platelet surface GPIIb-IIIa complex was analyzed by whole blood flow cytometry. CPB did not result in any significant change in the platelet surface expression of the GPIIb-IIIa complex, as determined by monoclonal antibodies (7E3, 10E5, and M148) directed against different epitopes near the fibrinogen binding site on the GPIIb-IIIa complex and a monoclonal antibody (Y2/51) directed against GPIIIa (Fig 4, panels A, B, C, and D).

Direct measurement of the binding of exogenous fibrinogen to platelets activated *in vitro* with ADP (reflecting exposure of the fibrinogen binding site on the GPIIb-IIIa complex[55]) demonstrated an increase in the K_D from 0.43 to 0.54 μM and no loss of binding sites during CPB (Fig 4, panel E).

Effect of CPB on Platelet Degranulation

The presence of activated platelets circulating in peripheral blood was investigated by the platelet binding of S12, a monoclonal antibody directed against P-selectin. Again, in order to avoid artefactual platelet activation during the separation of platelets, a whole blood flow cytometric assay was utilized.[41] CPB did not result in any significant overall increase in P-selectin expression on the surface of circulating platelets (Fig 5, panel A, closed circles). Analysis of the same data in terms of the percentage of circulating platelets that were P-selectin-positive resulted in a minimal (but statistically significant) increase during CPB. Thus, the percentage of circulating platelets that were P-selectin-positive were: $2.0 \pm 0.0\%$ (mean $\pm$ S.E.M., n = 14) preoperatively, $3.1 \pm 0.3\%$ at 45 minutes after the start of CPB ($p = 0.0054$ compared to preoperatively by paired Student t-test), and $2.9 \pm 0.3\%$ at the completion of CPB, immediately following the administration of protamine ($p = 0.0078$ compared to preoperatively).

Effect of CPB on Platelet Reactivity in Vitro

In the whole blood flow cytometric assay, CPB did not significantly affect the up-regulation of platelet surface of P-selectin induced *in vitro* by either PMA (Fig 5, panel A), the stable thromboxane A_2 analogue U46619 (Fig 6, panel A), or a combination of ADP and epinephrine (Fig 6, panel A). Similarly, CPB did not significantly affect the down-regulation of the platelet surface GPIb-IX complex or the up-regulation of the platelet surface GPIIb-IIIa complex induced *in vitro* by either PMA, U46619, or ADP/epinephrine (Fig 5, panel B and Fig 6, panels B and C). CPB did not alter the kinetics of up-regulation of P-selectin (Fig 7, panel A), down-regulation of GPIb-IX complex (Fig 7, panel B), or up-regulation of the GPIIb-IIIa complex (Fig 7, panel C).

Interestingly, administration of heparin appeared to augment both the PMA-induced up-regulation of P-selectin (Fig 5, panel A), and down-regulation of the GPIb-IX complex (Fig 5, panel B). Protamine sulfate administration appeared to reverse this augmentation (Fig 5, panels A and B).

In order to assess the reactivity of platelets to thrombin, the patients' platelets were washed free of heparin. CPB did not inhibit the thrombin-induced up-regulation of platelet surface P-selectin, up-regulation of the GPIIb-IIIa complex, or down-regulation of the GPIb-IX complex (Fig 8, panels A, B, C, and D). However, as expected, when the thrombin reactivity of platelets in whole blood containing the therapeutically administered heparin was measured, platelets were completely unreactive to thrombin at the following time points: 5 minutes after heparin administration, but before the start of CPB; after the start of CPB (normothermic conditions); beginning of maximal hypothermia on CPB; and 45 minutes after the start of CPB (hypothermic conditions) (data not shown).

Monoclonal antibody PAC1 does not bind to resting platelets, only to activated platelet that have exposed their fibrinogen binding site on the GPIIb-IIIa complex.[39] Therefore, the studies with PAC1 (Fig 8, panel D) demonstrate that a) CPB did not result in significant exposure of the fibrinogen binding site on the GPIIb-IIIa complex and b) CPB did not inhibit the ability of platelets to expose their fibrinogen receptors.

Effect of CPB on Platelet Reactivity in Vivo

In order to determine platelet reactivity *in vivo*, the time-dependent up-regulation of platelet surface P-selectin was determined by whole blood flow cytometry in the blood emerging from a standardized bleeding time wound (Fig 9). The previously reported[18,41] time-dependent up-regulation of platelet surface P-selectin was observed in samples obtained from bleeding time incisions

performed preoperatively ("PRE OP" time point in Fig 9) and after the start of anesthesia and surgery but before heparin and CPB ("PRE HEP" time point in Fig 9). However, this *in vivo* up-regulation of P-selectin was abolished 5 minutes after heparin administration but before the start of CPB ("HEP" time point in Fig 9) and 45 minutes after the start of CPB ("CPB 45" time point in Fig 9). The *in vivo* up-regulation of P-selectin returned to near normal within 2 hours after the completion of CPB ("POST 2" time point in Fig 9) and was completely normal within 24 hours after the completion of CPB ("POST 24" time point in Fig 9). The maximum up-regulation of P-selectin in shed blood collected during bypass was significantly decreased as compared to the mean values in shed blood collected before and after bypass (F statistic = 33.97, df = 2,28, $p < 0.001$, ANOVA for repeated measures).

These data demonstrate that *in vivo* platelet reactivity during CPB is markedly deficient (Fig 9), whereas *in vitro* platelet reactivity during CPB is normal (Figs 5, 6, 7, and 8). These contrasting *in vivo* and *in vitro* results were obtained on samples obtained from the same set of patients at the same time points using the same assays run in parallel with the same reagents.

Effect of CPB on Platelet Generation of Thromboxane A_2 In Vivo

To provide further evidence that platelet reactivity *in vivo* is deficient during CPB, thromboxane B_2 (a stable metabolite of thromboxane A_2) was assayed in the shed blood emerging from the standardized bleeding time wound. Generation of thromboxane B_2 in shed blood was reduced 5 minutes after heparin administration but before the start of CPB ("HEP" time point in Fig 10) and decreased further 45 minutes after the start of CPB ("CPB 45" time point in Fig 10). Generation of thromboxane B_2 in shed blood returned towards normal after the completion of CPB ("CPB END", "POST 2", and "POST 24" time points in Fig 10).

DISCUSSION

The most important factor contributing to the hemostatic defect associated with CPB is generally considered to be a platelet function defect.[1-4,56] However, the precise nature of the putative CPB-induced platelet function defect is controversial.[1,3]

Effect of CPB on the Platelet Surface GPIb-IX Complex

Some,[7,17,19] but not all,[20] previous studies have concluded that a CPB-induced decrease in platelet surface GPIb plays a role in the pathogenesis of the CPB-induced platelet dysfunction. GPIb is a receptor for von Willebrand factor and is important in the process of platelet adhesion to damaged blood vessel walls.[52] The study by George et al.[17] has been widely interpreted as evidence for a CPB-induced reduction in platelet surface GPIb. However, in George's study[17] all values for platelet surface GPIb during CPB were within or close to the normal range. van Oeveren et al.[19] reported a 25% reduction in GPIb during CPB, but, unlike the study by George et al.[17] and the present study, these investigators centrifuged and gel filtered the platelets prior to assay, thereby introducing the possibility of an artefactual *in vitro* decrease in platelet surface GPIb as a result of proteolysis[50] or activation.[49] Rinder et al.[7] reported a maximal reduction of platelet surface GPIb of 28%, but the only significant reduction in GPIb was at the completion of and after CPB. Thus, Rinder et al.[7] did not observe a significant reduction in platelet surface GPIb during CPB, when the bleeding time is most prolonged. Furthermore, carriers of Bernard-Soulier syndrome, an autosomal recessive inherited deficiency of platelet glycoproteins Ib, IX, and V, have a normal bleeding time and no hemorrhagic diathesis, despite an approximately 50% reduction in platelet surface GPIb.[51,57,58] Thus, the modest reductions in GPIb found by some investigators[7,19] do not provide a satisfactory

explanation either for the hemorrhagic diathesis or for the prolonged bleeding time associated with CPB.

We have recently developed a flow cytometric method that allows us to study the platelet surface expression of GPIb in whole blood, thereby avoiding potential artefactual reductions in platelet surface GPIb.[41] In the present study, as demonstrated by this whole blood assay, CPB did not result in a decrease in the platelet surface expression of the GPIb-IX complex (Fig 2). The possibility of a CPB-induced conformational change in the GPIb-IX complex, or a CPB-induced proteolysis of a fragment of the GPIb-IX complex, was excluded by the lack of change in the platelet binding of a panel of monoclonal antibodies (6D1, TM60, AK1, and FMC25) known to be directed against different epitopes on the GPIb-IX complex (Fig 2). Furthermore, CPB neither reduced the ristocetin-induced binding of von Willebrand factor to GPIb (Fig 2) nor increased the plasma concentration of glycocalicin, a proteolytic fragment of GPIb (Fig 3). Thus, by a combination of methods, the present study demonstrates that CPB is not associated with a loss of platelet surface GPIb.

Effect of CPB on the Platelet Surface GPIIb-IIIa Complex

Some,[7,13,16,17] but not other,[18,19] previous studies have concluded that a CPB-induced decrease in the platelet surface GPIIb-IIIa complex plays a role in the pathogenesis of the CPB-induced platelet dysfunction. The GPIIb-IIIa complex is a receptor for fibrinogen and is important in the process of platelet aggregation.[55] Using washed platelet methods, Wenger et al.[13] reported a decrease of 25% in the whole platelet GPIIIa content during CPB and a decrease of 41% in the platelet binding of exogenous fibrinogen. Dechavanne et al.[16] also used a washed platelet method and reported a 32% decrease in the platelet surface GPIIb-IIIa complex during CPB. Utilizing a whole blood method,

George et al.[17] reported a slight decrease in platelet surface GPIIb during CPB. However, in this study[17] all values for platelet surface GPIIb during CPB were within or close to the normal range. Rinder et al.,[7] using a whole blood method, reported a 21% (but statistically insignificant) decrease in the platelet surface GPIIb-IIIa complex after CPB. Furthermore, carriers of Glanzmann's thrombasthenia, an autosomal recessive inherited deficiency of the platelet GPIIb-IIIa complex, have a normal bleeding time and no hemorrhagic diathesis, despite an approximately 50% reduction in the platelet surface GPIIb-IIIa complex.[57,59] Therefore, the quantitatively more modest reductions in the platelet surface GPIIb-IIIa complex found during CPB by some investigators[7,13,16,17] do not provide a satisfactory explanation for the prolonged bleeding time and hemorrhagic diathesis associated with CPB. Moreover, van Oeveren et al.[19] detected a modest increase in both platelet surface GPIIb-IIIa and ADP-induced fibrinogen binding during CPB. Finally, utilizing a whole blood flow cytometric method, Abrams et al.[18] reported that fibrinogen binding to the platelet GPIIb-IIIa complex was slightly increased during CPB, as determined by the binding of a monoclonal antibody (PAC1) directed against the fibrinogen binding site on the GPIIb-IIIa complex and by the binding of a monoclonal antibody (9F9) directed against platelet-bound fibrinogen.

In the present study, as demonstrated by a whole blood flow cytometric assay, CPB did not result in a decrease in the platelet surface expression of the GPIIb-IIIa complex (Fig 4). The possibility of a CPB-induced conformational change in the GPIIb-IIIa complex, or a CPB-induced proteolysis of a fragment of the GPIIb-IIIa complex, was excluded by the lack of change in the platelet binding of a panel of monoclonal antibodies (7E3, 10E5, M148, and Y2/51) known to be directed against different epitopes on the GPIIb-IIIa complex (Fig 4). In addition, we directly studied the binding of exogenous fibrinogen to the GPIIb-IIIa complex and found no CPB-induced reduction (Fig 4). Finally, we demonstrated that irrespective of whether peripheral blood samples were drawn

before, during, or after CPB, maximal *in vitro* stimulation of washed platelets by thrombin resulted in the same exposure of the fibrinogen binding site on the GPIIb-IIIa complex, as determined by monoclonal antibody PAC1 (Fig 8, panel D). Thus, by a combination of methods, the present study demonstrates that CPB is not associated with a loss of the platelet surface GPIIb-IIIa complex.

Effect of CPB on Platelet Degranulation

Plasma assays of ß-thromboglobulin (ß-TG) and platelet factor 4 (PF4) have been widely used as markers of platelet degranulation and secretion.[60] Most studies have reported increased plasma concentrations of ß-TG and PF4 during and after CPB.[4,6,15,61-63] Some,[62] but not other,[64] studies have found that platelet levels of ß-TG were reduced by CPB and concluded that degranulation had occurred during CPB. However, there are two major problems with the use of plasma assays of ß-TG and PF4 to monitor platelet degranulation and secretion. First, separation of plasma from platelets prior to the performance of these assays frequently results in artefactual *in vitro* degranulation and secretion.[60] Second, elevated plasma concentrations of plasma ß-TG and PF4 reflect not only the number of circulating degranulated platelets but also lysed platelets and non-circulating degranulated platelets adherent to synthetic surfaces or vessel walls.[3,15]

Electron microscopic studies of platelet degranulation during CPB are also fraught with the possibility of artefactual *in vitro* platelet activation. Using electron microscopy, Harker et al.[4] found that platelets circulating during CPB had partially released their α granules but not their dense granules, whereas neither Zilla et al.[15] nor Dechavanne et al.[16] found CPB-induced degranulation of circulating platelets.

More recently, a number of investigators have studied the effect of CPB on the binding of monoclonal antibodies directed against granule antigens that are only present on the platelet surface

after degranulation.[7,14,16-18] Utilizing washed platelet systems, Nieuwenhuis et al.[14] found a modest increase during CPB of the platelet binding of a monoclonal antibody directed against a 53 kDa lysosomal antigen, whereas Dechavanne et al.[16] found that CPB did not result in an increase in the platelet surface expression of the α granule membrane protein P-selectin (also known as GMP-140 and PADGEM protein). Utilizing whole blood methods that are much less likely than washed platelet methods to result in artefactual *in vitro* platelet degranulation,[65] both George et al.[17] and Abrams et al.[18] demonstrated that CPB resulted in no increase in the platelet surface expression of P-selectin. Although Rinder et al.[7] used a whole blood flow cytometric assay and found a 29% increase in P-selectin-positive platelets at the end of CPB, the amount of increase in P-selectin on the P-selectin-positive platelets was modest.

Consistent with these previous studies, the present whole blood flow cytometric study of peripheral blood samples during CPB revealed minimal P-selectin expression on the platelet surface (Fig 5, panel A, closed circles). This virtual absence of circulating degranulated platelets suggests that the previously reported CPB-induced increase in plasma concentrations of ß-TG and PF4[4,6,15,61-63] originates from either a) degranulated platelets that are very rapidly cleared from the circulation[66] (possibly by circulating monocytes and neutrophils[67,68]), b) non-circulating degranulated platelets adherent to synthetic surfaces or vessel walls, and/or c) platelet lysis *in vivo*[15] or *in vitro*.

Effect of CPB on Platelet Reactivity in Vitro

The results of studies of the effect of CPB on platelet aggregation, as determined by standard nephelometric methods, are inconsistent.[4-12,15,69] Although many of these studies describe CPB-induced defects in platelet aggregation, the reported responses to known platelet agonists are widely variable. Some of the reported defects are modest[15,69] and some investigators did not detect CPB-

induced defects in platelet aggregation.[12,13] These inconsistencies probably result in part from the fact that platelet aggregation, especially in a complex clinical setting, is semiquantitative and subject to standardization problems.[70,71] Furthermore, most of the reported platelet aggregometry studies during CPB were performed in platelet-rich plasma without normalizing the platelet count. The CPB-induced "platelet aggregation defect" may therefore simply reflect the CPB-induced thrombocytopenia.

In this study, in contrast to all previous studies, platelet reactivity *in vitro* was analyzed not by aggregometry but by whole blood flow cytometric assay of peripheral blood samples obtained before, during, and after CPB. CPB did not result in a defect in platelet reactivity *in vitro*, as determined by agonist-induced up-regulation of the platelet surface P-selectin (monitored by monoclonal antibody S12), up-regulation of the fibrinogen binding site on the GPIIb-IIIa complex (monitored by monoclonal antibodies PAC1 and 7E3), and down-regulation of the GPIb-IX complex (monitored by monoclonal antibody 6D1) (Figs 5, 7, and 8). The results were the same irrespective of whether the *in vitro* platelet agonist was PMA, the stable thromboxane A_2 agonist U46619, a combination of ADP and epinephrine, or, in a washed platelet system, thrombin (Figs 5, 6, 7, and 8), thereby excluding the possibility of a CPB-induced signal transduction defect. One could speculate that the heparin-induced augmentation of platelet reactivity *in vitro* (Fig 5), which was reversed by protamine sulfate administration (Fig 5), might provide a specific mechanism through which platelets may be particularly prone to artefactual *in vitro* activation during the separation of platelet-rich plasma and therefore appear to be less reactive to exogenous agonists in platelet aggregation studies.

Effect of CPB on Platelet Reactivity in Vivo

No previous study of CPB has attempted to directly assess the effect of platelet reactivity *in vivo*. This was accomplished in the present study by two assays of the shed blood emerging from a standardized *in vivo* bleeding time wound.[18,41]

First, as determined by whole blood flow cytometry, the previously described[18,41] time-dependent up-regulation of the platelet surface expression of P-selectin was observed in shed blood samples obtained from bleeding time incisions performed preoperatively (Fig 9). However, this *in vivo* up-regulation of P-selectin was abolished a) 5 minutes after heparin administration but before the start of CPB and b) 45 minutes after the start of CPB (Fig 9). The *in vivo* up-regulation of P-selectin returned to near normal within 2 hours of the completion of CPB and completely normal within 24 hours after the completion of CPB (Fig 9).

Second, as determined by radioimmunoassay, generation of thromboxane B_2 (a stable metabolite of thromboxane A_2 and an important marker of platelet activation[72]) was reduced in shed blood samples during CPB (Fig 10).

Thus, by a combination of methods, the present study demonstrates that, in striking contrast to the normal *in vitro* platelet reactivity observed during CPB (see above), *in vivo* platelet reactivity during CPB is markedly deficient.

The Platelet Function Defect of CPB

CPB is associated with a hemostatic defect, the hallmark of which is a prolonged bleeding time.[1-4] A platelet function defect appears to be the basis for this prolongation of the bleeding time during CPB, because: i) the modest decrease in the platelet count during CPB is insufficient to account for the marked prolongation of the bleeding time during CPB (Fig 1, panel B, and ref. 73);

ii) the relationship between bleeding time and degree of thrombocytopenia is discordant at the completion of CPB and after CPB (Fig 1, panels A and B, and ref. 4); iii) von Willebrand factor levels remain within or above the normal range during and after CPB (Fig 1, panel C and refs. 69,74,75); iv) up-regulation of platelet surface P-selectin *in vivo* is abolished during CPB (Fig 9); and v) *in vivo* generation of thromboxane A_2, an important marker of platelet activation,[72] is deficient during CPB (Fig 10).

However, the hypothesis that CPB results in an intrinsic platelet function defect is not supported by the present findings during and after CPB: a) normal antigenic and functional expression of the platelet surface GPIb-IX and GPIIb-IIIa complexes, b) absence of circulating degranulated platelets, and c) normal platelet reactivity *in vitro*. Taken together with these findings, the demonstration of a CPB-induced deficiency of platelet reactivity *in vivo* suggests that the "platelet function defect" of CPB is the result of a defect extrinsic to the platelet, such as an *in vivo* lack of availability of platelet agonists. Thrombin, the preeminent platelet agonist *in vivo*,[76-78] is functionally deficient during CPB because of the presence of high circulating concentrations of heparin. Although thrombin bound to fibrin clots is relatively protected from inhibition by heparin-antithrombin III,[79,80] the high concentrations of heparin used during CPB (4.5 U/mL, with an ACT greater than or equal to 999 seconds, in this study) are sufficient to completely overcome this protection.[79] The importance of heparin inhibition of thrombin in the extrinsic platelet function defect of CPB is suggested by the finding that 5 minutes after heparin administration but before the start of CPB: a) the *in vivo* up-regulation of platelet surface P-selectin was abolished ("HEP" time point in Fig 9), b) the *in vivo* generation of thromboxane A_2 was almost maximally inhibited ("HEP" time point in Fig 10), c) the bleeding time was prolonged ("HEP" time point in Fig 1) and d) platelets in whole blood were completely unreactive to thrombin *in vitro* (data not shown).

Heparin is nearly universally used during CPB. The present study provides evidence for two distinct effects of heparin on platelet function during CPB. First, heparin augments platelet activation in whole blood exposed to an *exogenous* platelet agonist *in vitro* (figure 5). Second, as discussed above, heparin suppresses platelet activation *in vivo* via inhibition of *endogenous* thrombin. These two apparently opposite effects of heparin on platelets are neither inconsistent nor contradictory. Thus, although heparin augments the activatability of platelets, the platelets are not in fact activatable *in vivo* because thrombin, the preeminent agonist,[76-78] is unavailable.

Neither the presence of fibrin degradation products nor the use of hypothermia can account for the differences between platelet function *in vitro* and *in vivo* observed in the present study. Inhibition of platelet function by circulating fibrin(ogen) degradation products[81] does not account for our findings because platelet activation *in vitro* was normal. Although hypothermia is likely to play a role in the extrinsic platelet function defect associated with CPB,[82-84] hypothermia does not account for the differences between platelet activatibility *in vitro* and *in vivo*, because our *in vitro* studies were performed at 22°C.

In summary, CPB resulted in: 1) no loss of the platelet surface GPIb-IX complex, 2) no loss of the platelet surface GPIIb-IIIa complex, 3) a minimal number of circulating degranulated platelets, 4) normal platelet reactivity *in vitro*, and 5) markedly deficient platelet reactivity *in vivo*. These data suggest that the "platelet function defect" of CPB is not a defect intrinsic to the platelet but is an extrinsic defect such as an *in vivo* lack of availability of platelet agonists. The near universal use of heparin during CPB is likely to contribute substantially to this defect via its inhibition of thrombin.

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FIGURE LEGENDS

Fig 1. Effect of CPB on the bleeding time (panel A), platelet count (panel B), and plasma von Willebrand factor concentration (panel C). The abbreviations listed on the horizontal axis refer to the blood sampling time points before, during, and after CPB, as defined in Table 1. Data are mean $\pm$ S.E.M., n = 16 separate patients.

Fig 2. Effect of CPB on the platelet surface expression of the GPIb-IX complex. The abbreviations listed on the horizontal axis refer to the perioperative blood sampling time points, as defined in Table 1. Panels A through D: the platelet binding of a panel of GPIb-IX-specific monoclonal antibodies, as determined by whole blood flow cytometry. The antibodies are directed against the von Willebrand factor binding site on GPIb (6D1), the thrombin binding site on GPIb (TM60), GPIX (FMC25), and the GPIb-IX complex (AK1). Panel E: ristocetin-induced binding of exogenous von Willebrand factor (vWf) to washed platelets, as determined by flow cytometry with a polyclonal anti-vWf antibody. In each panel, antibody binding at the preoperative ("PRE OP") time point was assigned a value of 100 units of fluorescence. Data are mean $\pm$ S.E.M. n = 4.

Fig 3. Effect of CPB on the plasma concentration of glycocalicin (a proteolytic fragment of GPIb). The abbreviations listed on the horizontal axis refer to the perioperative blood sampling time points, as defined in Table 1. Plasma glycocalicin (solid line) was measured by a competitive inhibition assay. In order to assess the effect of hemodilution during the bypass procedure, hematocrit (dotted line) was also measured. Data are mean $\pm$ S.E.M. n = 16.

Fig 4. Effect of CPB on the platelet surface expression of the GPIIb-IIIa complex. The abbreviations listed on the horizontal axis refer to the perioperative blood sampling time points, as defined in Table 1. Panels A through D: the platelet binding of a panel of GPIIb-IIIa-specific monoclonal antibodies, as determined by whole blood flow cytometry. Antibodies 7E3, 10E5, and M148 are directed against different epitopes near the fibrinogen binding site on the GPIIb-IIIa complex. Y2/51 is directed against the GPIIIa subunit. In each panel, antibody binding at the preoperative ("PRE OP") time point was assigned a value of 100 units of fluorescence. Panel E: binding of exogenous radioiodinated fibrinogen to ADP-stimulated washed platelets. Fibrinogen binding is expressed as molecules per platelet. Data are mean $\pm$ S.E.M., n = 4.

Fig 5. Effect of CPB on platelet reactivity to PMA, as determined by whole blood flow cytometry. The abbreviations listed on the horizontal axis refer to the perioperative blood sampling time points, as defined in Table 1. Blood samples were incubated with PMA 0 (solid circles), 0.25 (solid triangles) or 10 μM (solid squares). Panel A: platelet surface expression of platelet-selectin, as determined by monoclonal antibody S12. The fluorescence intensity of platelets incubated with PMA 10 μM at the preoperative ("PRE OP") time point was assigned a value of 100 units. Panel B: platelet surface GPIb, as determined by monoclonal antibody 6D1. The fluorescence intensity of platelets incubated without PMA at the preoperative ("PRE OP") time point was assigned a value of 100 units. Data are mean $\pm$ S.E.M. n = 16.

Fig 6. The effect of CPB on platelet reactivity to the stable thromboxane A_2 analogue U46619 and to a combination of ADP and epinephrine (Epi), as determined by whole blood flow cytometry. The abbreviations listed on the horizontal axis refer to the perioperative blood sampling time points, as defined in Table 1. Blood samples were incubated with the indicated agonist, and the platelet binding measured of monoclonal antibodies S12 (P-selectin-specific) (panel A), 6D1 (GPIb-specific) (panel B), and 7E3 (GPIIb-IIIa complex-specific) (panel C). For assays with S12 and 7E3, the fluorescence intensity of platelets incubated with 1 U/mL thrombin at the preoperative ("PRE OP") time point was assigned a value of 100 units. For assays with 6D1, the fluorescence intensity of platelets without added exogenous agonist at the preoperative ("PRE OP") time point was assigned a value of 100 units. Data are mean $\pm$ S.E.M., n = 4.

Fig 7. The effect of CPB on the kinetics of the up-regulation of P-selectin and the GPIIb-IIIa complex and the down-regulation of GPIb. Blood samples were drawn at the perioperative time points listed in the small box and fully defined in Table 1. The samples were incubated with U46619 at 22°C for various lengths of time, immediately fixed with 1% formaldehyde, and analyzed by whole blood flow cytometry. For assays with S12 (panel A) and 7E3 (panel C), the fluorescence intensity of platelets incubated for 10 minutes with 1 U/mL thrombin at the preoperative ("PRE OP") time point was assigned a value of 100 units. For assays with 6D1 (panel B), the fluorescence intensity of platelets without added exogenous agonist at the preoperative ("PRE OP") time point was assigned a value of 100 units. Data are mean $\pm$ S.E.M. n = 4.

Fig 8. The effect of CPB on the thrombin reactivity of platelets. The abbreviations listed on the horizontal axis refer to the perioperative blood sampling time points, as defined in Table 1. The platelets were washed and incubated with the indicated thrombin concentrations. Thrombin-induced up-regulation of the platelet surface expression of P-selectin (panel A) and the GPIIb-IIIa complex (panels C and D) and down-regulation of GPIb (panel B) was measured by flow cytometry. For assays with S12 (panel A), 7E3 (panel C), and PAC1 (panel D), the fluorescence intensity of platelets incubated with thrombin 1 U/mL at the preoperative ("PRE OP") time point was assigned a value of 100 units. For assays with 6D1 (panel B), the fluorescence intensity of platelets without added exogenous thrombin at the preoperative ("PRE OP") time point was assigned a value of 100 units. Data are mean $\pm$ S.E.M. n = 4.

Fig 9. The effect of CPB on activation-induced up-regulation of the platelet surface expression of P-selectin in whole blood *in vivo*. Blood samples were drawn at the perioperative time points listed to the right of each panel and fully defined in Table 1. A standardized bleeding time was performed and, without touching the wound, the shed blood was collected with a micropipet directly from the wound site at 2 minute intervals (as shown on the horizontal axis) until the cessation of bleeding. The sample was immediately fixed and then analyzed by whole blood flow cytometry with monoclonal antibody S12. The maximal binding of S12 to platelets at the preoperative ("PRE OP") time point was assigned a value of 100 units of fluorescence. This experiment is representative of 9 separate experiments.

Fig 10. The effect of CPB on thromboxane B_2 generation in response to a standardized vascular injury *in vivo*. The abbreviations listed on the horizontal axis refer to the perioperative time points, as defined in Table 1. At each of these perioperative time points, a standardized bleeding time wound was performed and the shed blood collected every 30 seconds until a 600 μL aliquot of blood was obtained. The thromboxane B_2 concentration of the aliquot was determined by radioimmunoassay. Data are mean $\pm$ S.E.M. n = 16.

Table 1. Blood sampling time points before, during, and after CPB.

PRE OP	Preoperative.
PRE HEP	After the start of anesthesia and surgery, but before heparin and CPB.
HEP	5 minutes after heparin administration, but before the start of CPB.
CPB NT	After the start of CPB (normothermic conditions).
CPB HT	Beginning of maximal hypothermia on CPB.
CPB 45	45 minutes after the start of CPB (hypothermic conditions).
CPB END	Completion of CPB, immediately following administration of protamine.
POST 2	2 hours after completion of CPB.
POST 24	24 hours after completion of CPB.
POST 48	48 hours after completion of CPB

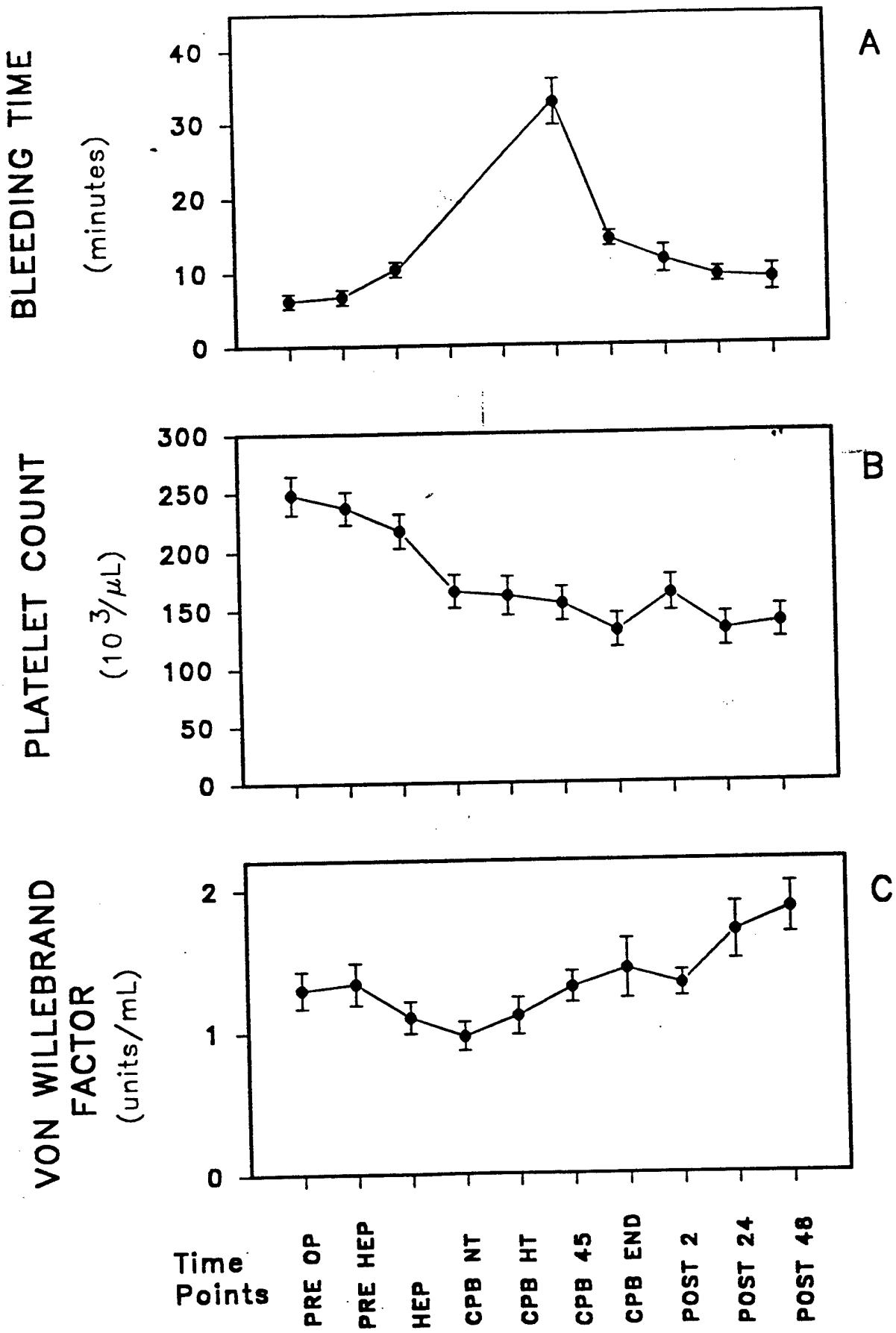
BLEEDING TIME
(minutes)
40
30
20
10
0
A
PLATELET COUNT
($10^3/\mu L$)
300
250
200
150
100
50
0
B
VON WILLEBRAND FACTOR
(units/mL)
2
1
0
C
Time Points
PRE OP
PRE HEP
HEP
CPB NT
CPB HT
CPB 45
CPB END
POST 2
POST 24
POST 48

Fig. 1

GPIb/IX

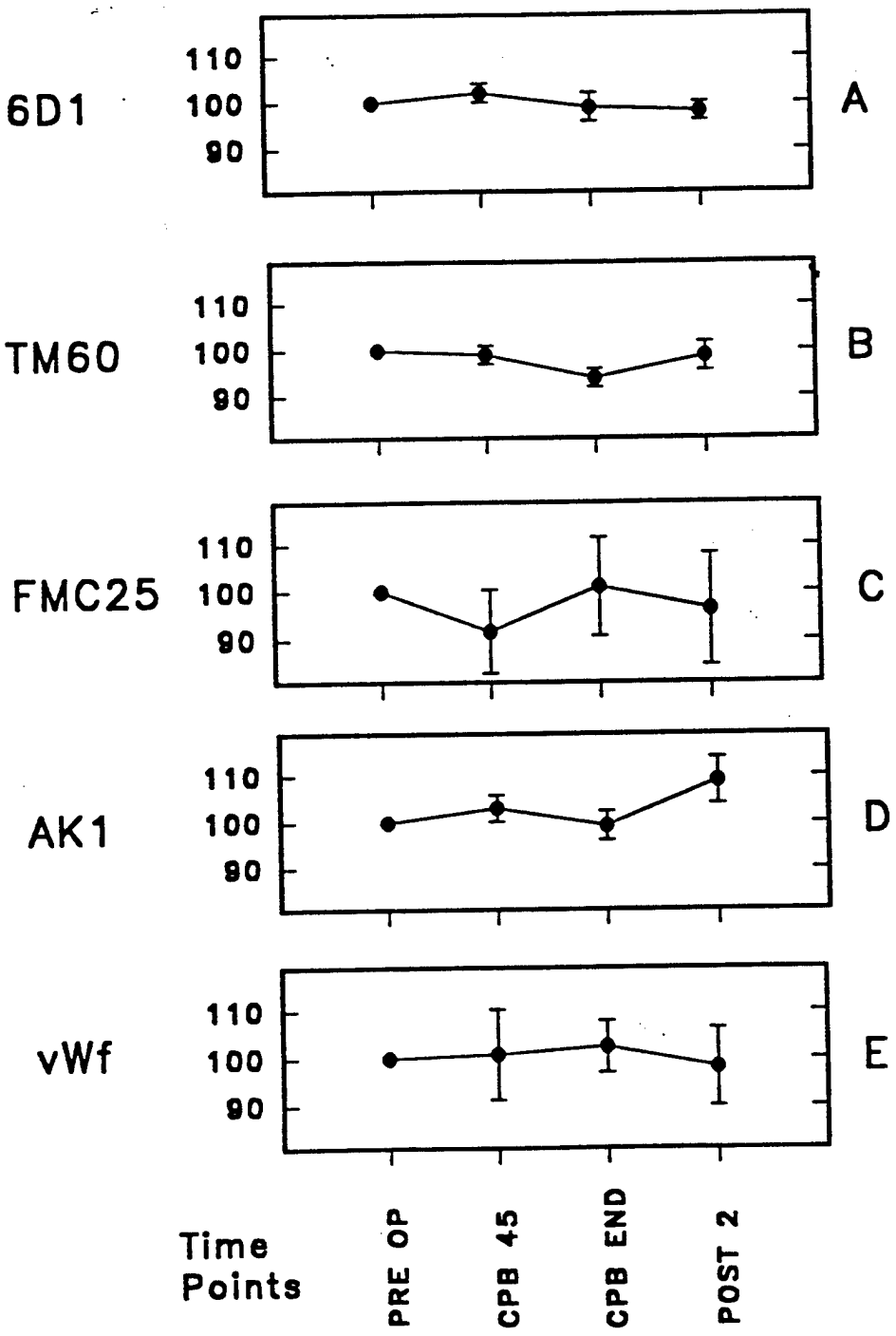
6D1
110
100
90
A
TM60
110
100
90
B
FMC25
110
100
90
C
AK1
110
100
90
D
vWf
110
100
90
E
Time Points
PRE OP
CPB 45
CPB END
POST 2

Fig. 2

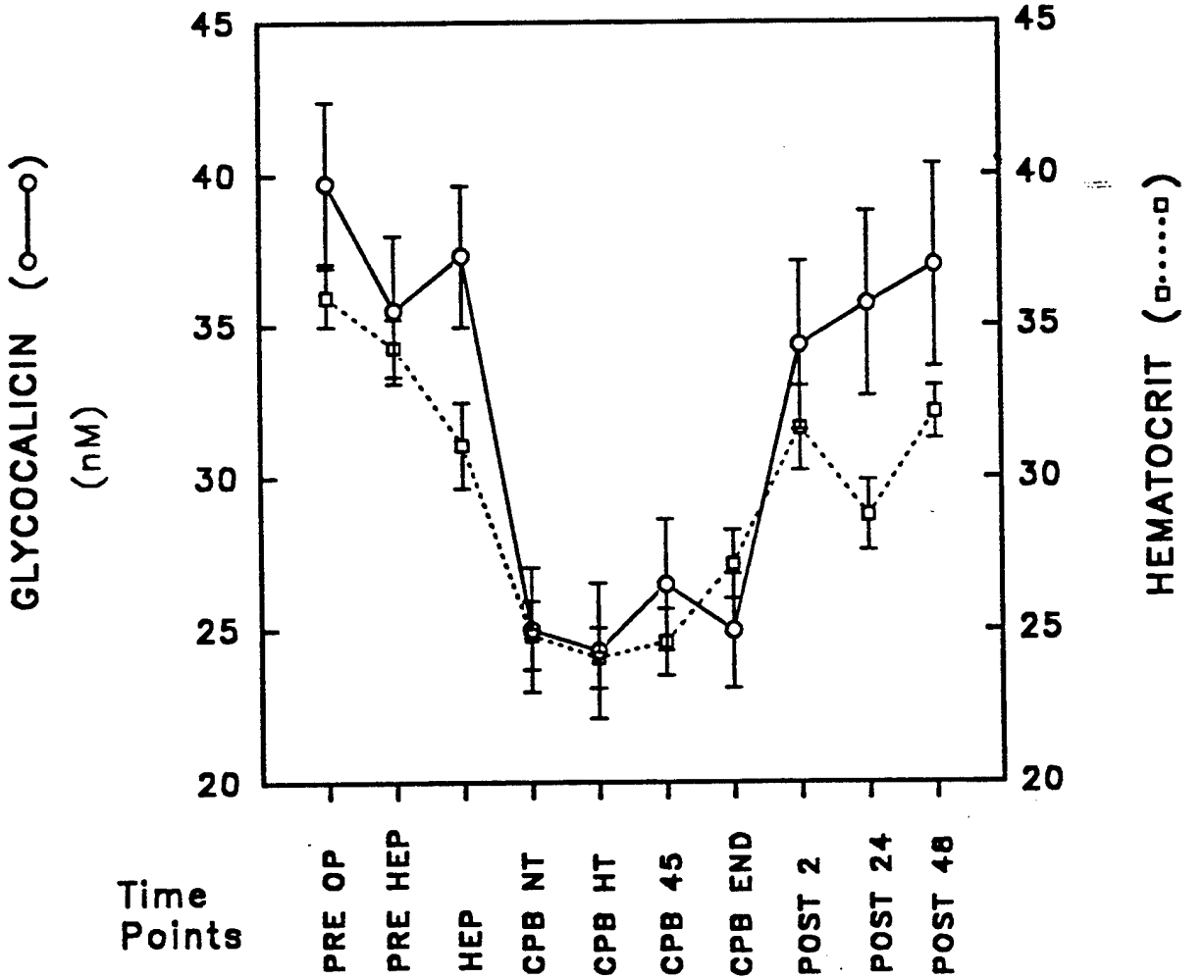
GLYCOCALICIN (o—o)
(nM)
HEMATOCRIT (□.....□)
(%)
45
40
35
30
25
20
Time Points
PRE OP
PRE HEP
HEP
CPB NT
CPB HT
CPB 45
CPB END
POST 2
POST 24
POST 48

Fig.3

GPIIb/IIIa

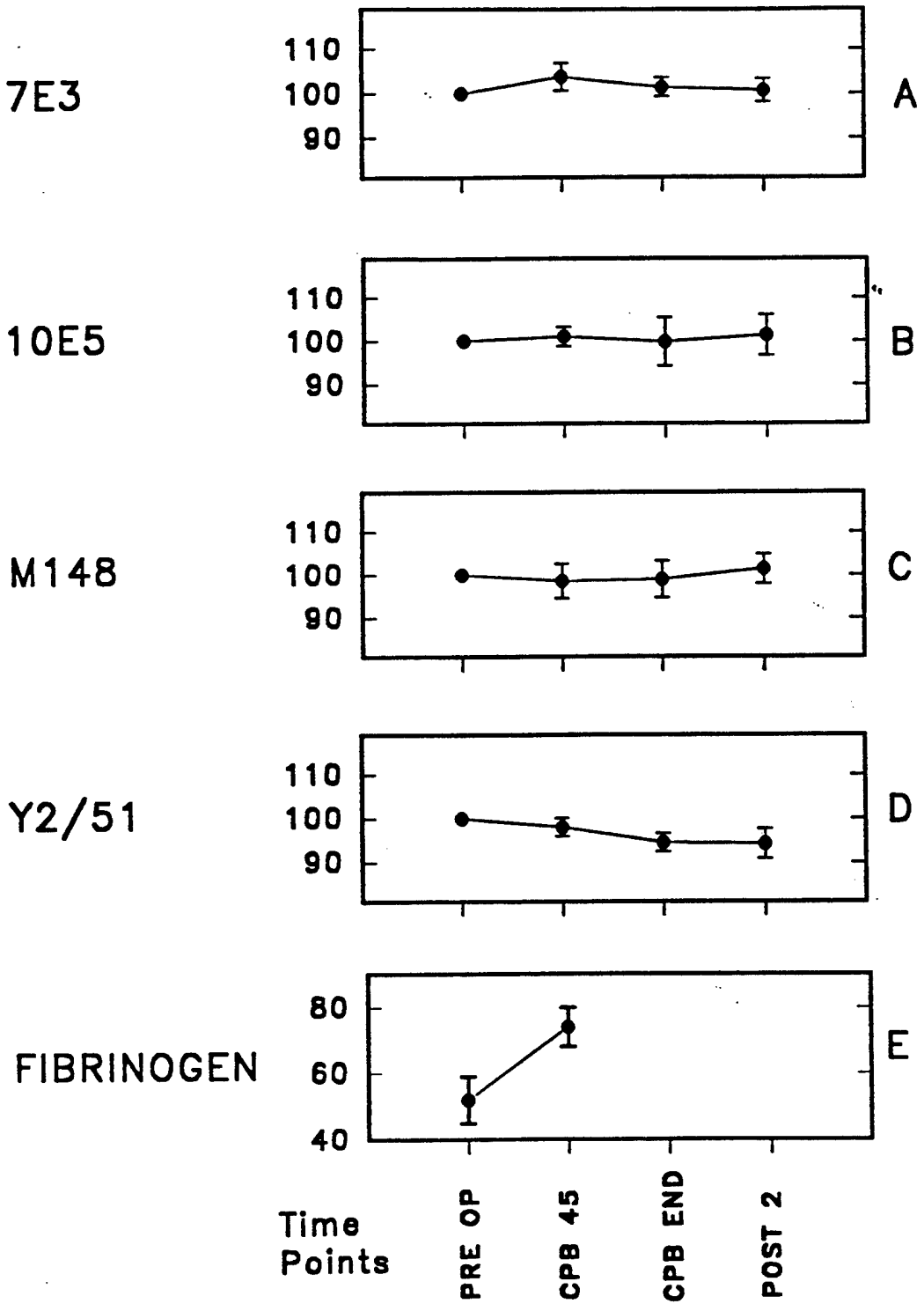

Fig. 4

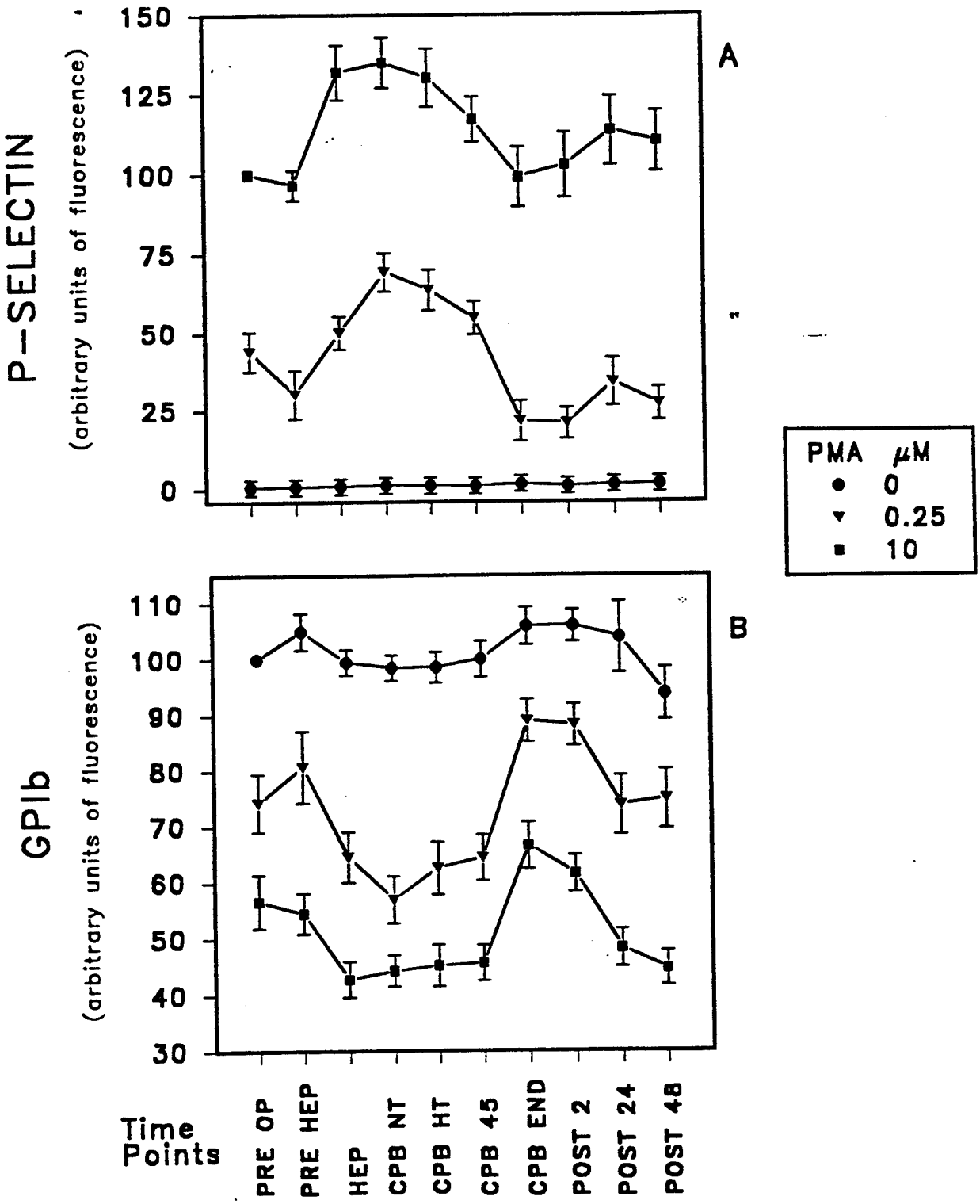
P-SELECTIN
(arbitrary units of fluorescence)
150
125
100
75
50
25
0
A
PMA μM
0
0.25
10
GPIb
(arbitrary units of fluorescence)
110
100
90
80
70
60
50
40
30
B
Time Points
PRE OP
PRE HEP
HEP
CPB NT
CPB HT
CPB 45
CPB END
POST 2
POST 24
POST 48

Fig. 5

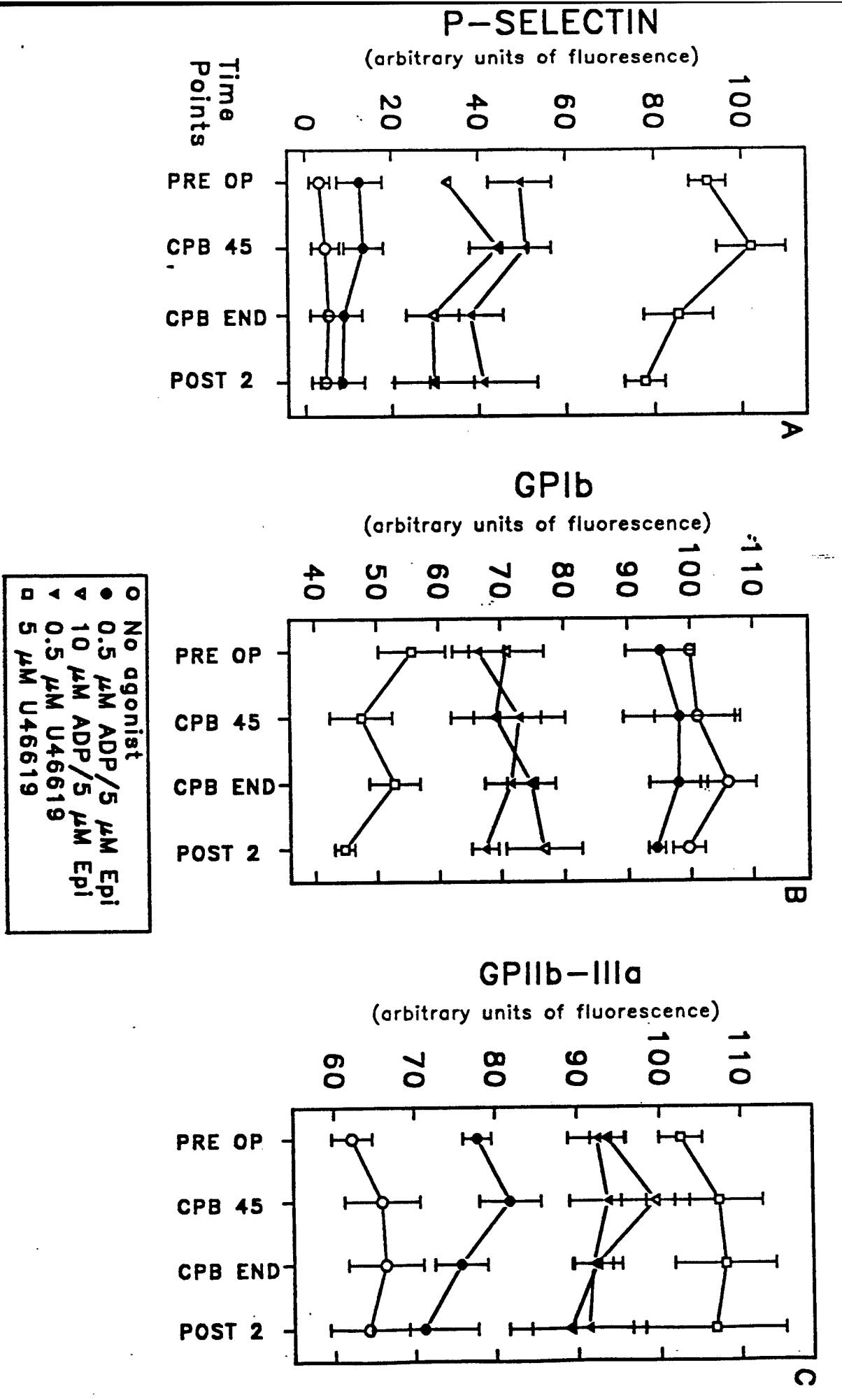
P-SELECTIN
(arbitrary units of fluoresence)
Time Points
0
20
40
60
80
100
PRE OP
CPB 45
CPB END
POST 2
A
GPIb
(arbitrary units of fluorescence)
40
50
60
70
80
90
100
110
PRE OP
CPB 45
CPB END
POST 2
B
GPIIb-IIIa
(arbitrary units of fluorescence)
60
70
80
90
100
110
PRE OP
CPB 45
CPB END
POST 2
C
o No agonist
● 0.5 μM ADP/5 μM Epi
▲ 10 μM ADP/5 μM Epi
▼ 0.5 μM U46619
□ 5 μM U46619

Fig. 6

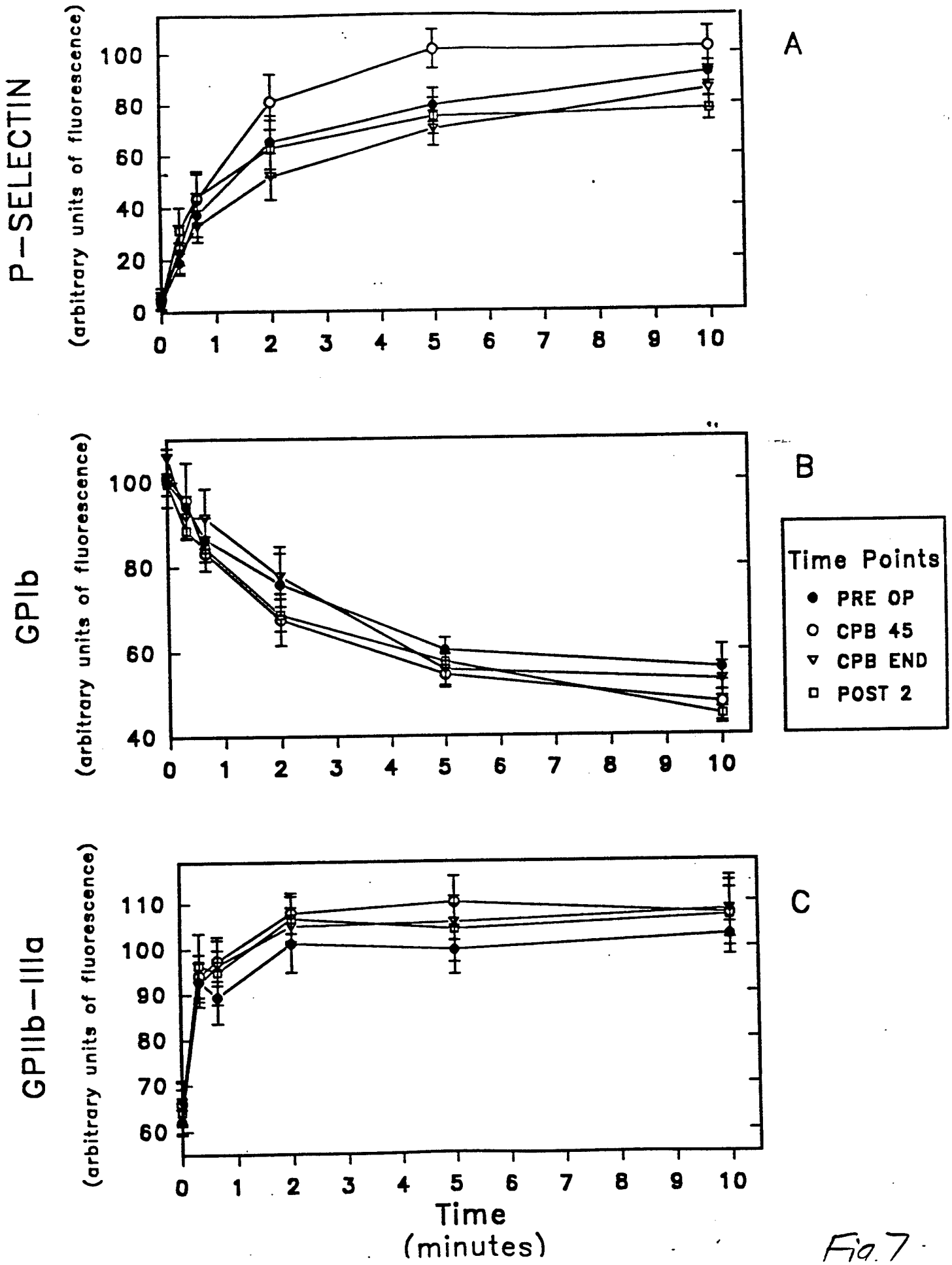
P-SELECTIN
(arbitrary units of fluorescence)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
A
GPIb
(arbitrary units of fluorescence)
100
80
60
40
0 1 2 3 4 5 6 7 8 9 10
B
Time Points
PRE OP
CPB 45
CPB END
POST 2
GPIIb-IIIa
(arbitrary units of fluorescence)
110
100
90
80
70
60
0 1 2 3 4 5 6 7 8 9 10
Time
(minutes)
C

Fig. 7

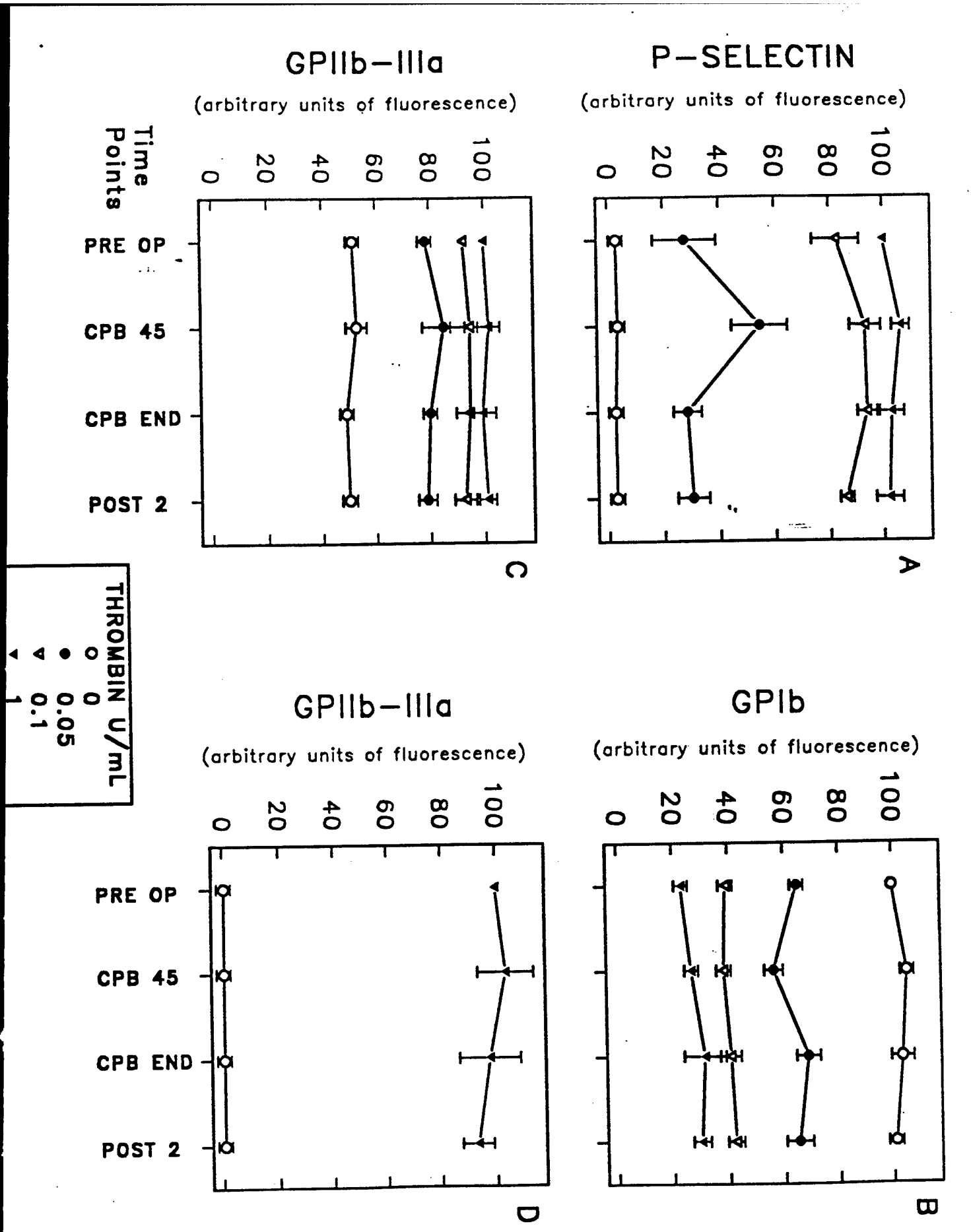
GPIIb–IIIa
(arbitrary units of fluorescence)
P–SELECTIN
(arbitrary units of fluorescence)
Time Points
PRE OP
CPB 45
CPB END
POST 2
0
20
40
60
80
100
C
A
THROMBIN U/mL
0
0.05
0.1
1
GPIIb–IIIa
(arbitrary units of fluorescence)
GPIb
(arbitrary units of fluorescence)
D
B

Fig. 8

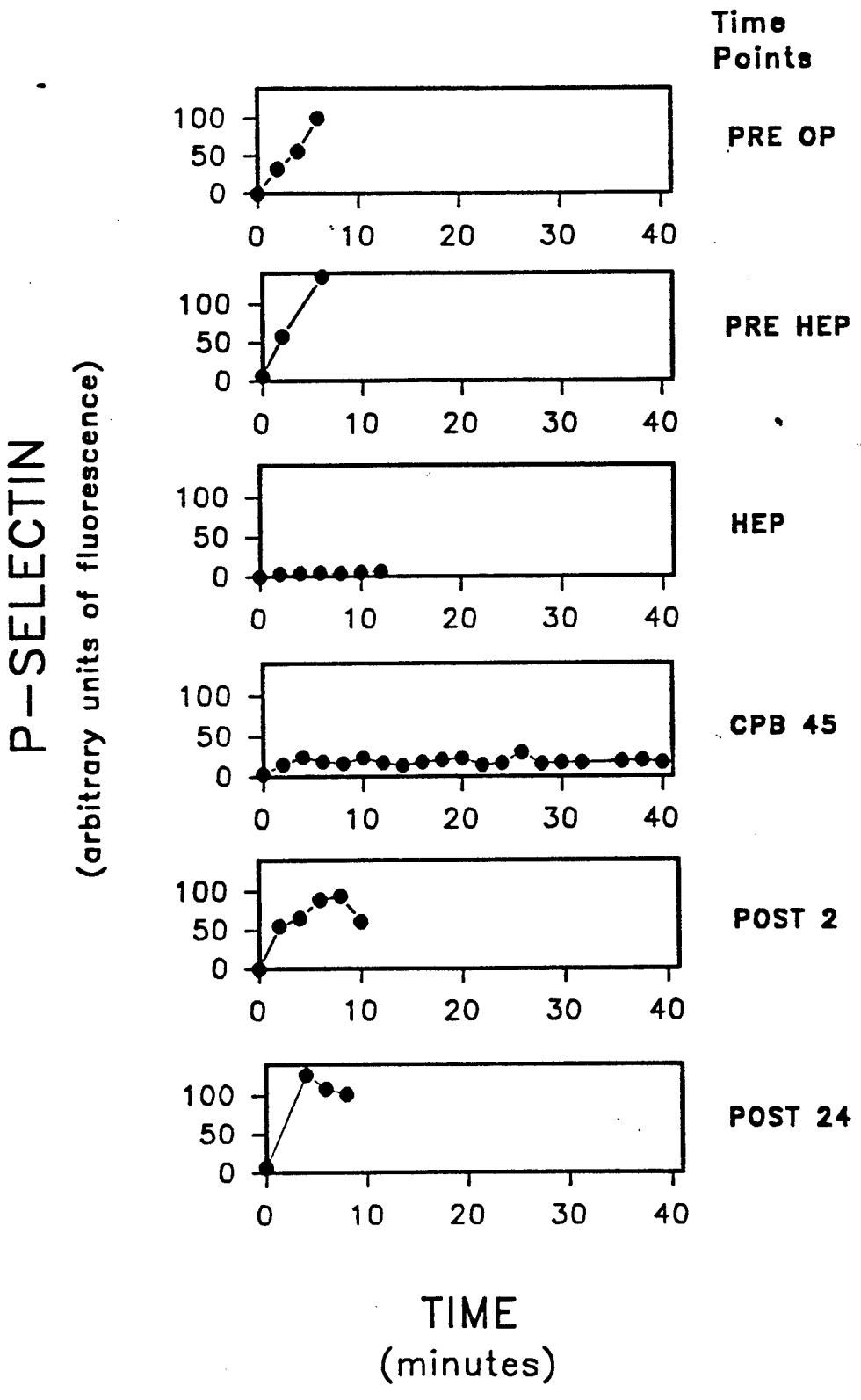
Time Points
PRE OP
PRE HEP
HEP
CPB 45
POST 2
POST 24
100
50
0
0
10
20
30
40
P—SELECTIN
(arbitrary units of fluorescence)
TIME
(minutes)

Fig. 9

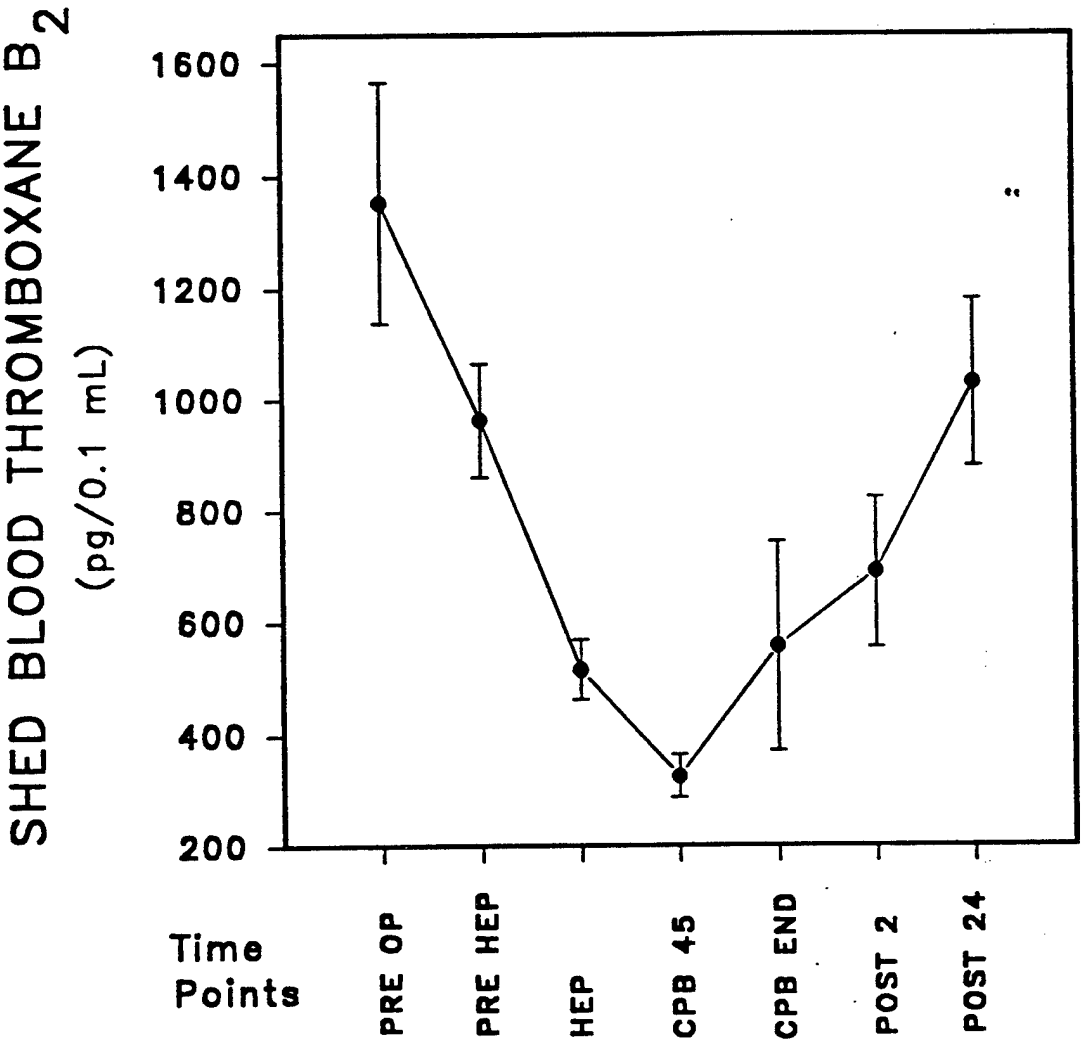
SHED BLOOD THROMBOXANE B_2
(pg/0.1 mL)
1600
1400
1200
1000
800
600
400
200
Time Points
PRE OP
PRE HEP
HEP
CPB 45
CPB END
POST 2
POST 24

Fig. 10